Food Microbiology 43 (2014) 20–27

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Effects of nitrogen and potassium fertilization on the susceptibility of tomatoes to post-harvest proliferation of *Salmonella enterica*

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form
21 March 2014
Accepted 22 March 2014
Available online 9 May 2014

Keywords:
Salmonella enterica
Post-harvest infection
Produce safety
Field production conditions
Secondary metabolite

ABSTRACT

Fresh fruits and vegetables are increasingly recognized as vehicles of salmonellosis. Pre- and post-harvest environmental conditions, and physiological, and genetic factors are thought to contribute to the ability of human pathogens to persist in the production environment, attach to, colonize and proliferate in and on raw produce. How field production conditions affect the post-harvest food safety outcomes is not entirely understood. This study tested how varying nitrogen and potassium fertilization levels affected the "susceptibility" of tomatoes to *Salmonella* infections following the harvest of fruits. Two tomato varieties grown over three seasons under high, medium, and low levels of nitrogen and potassium fertilization in two locations were inoculated with seven strains of *Salmonella*. Even though the main effects of nitrogen and potassium fertilization on the susceptibility of tomatoes to infections with *Salmonella enterica* were not statistically significant overall, differences in nitrogen concentrations in plant tissues correlated with the susceptibility of partially ripe tomatoes (cv. Solar Fire) to *Salmonella*. Tomato maturity and the season in which tomatoes were produced had the strongest effect on the ability of *Salmonella* to multiply in tomatoes. Tomato phenolics, accumulation of which is known to correlate with rates of the N fertilization, did not inhibit growth of *Salmonella in vitro*.

1. Introduction

The recurrent outbreaks of gastroenteritis linked to the consumption of fresh fruits and vegetables and caused by strains of non-typhoidal *Salmonella* and enterovirulent *Escherichia coli* suggest that these human pathogens can contaminate produce at any stage of the production cycle, farm to fork. Outbreaks of gastroenteritis associated with the consumption of vegetables have been on the rise, while those linked to the traditional sources (like fresh eggs) have been declining (Gould et al., 2013; Kozak et al., 2013). Over the past decade, produce-linked outbreaks of human illness resulted in thousands of hospitalizations and multi-million dollar damage to the industry (Gould et al., 2013; Kozak et al., 2013; Mandrell, 2009).

Significant progress has been made in understanding the ecology of human pathogens in the crop production environment. The same *Salmonella enterica* strains were isolated from environmentally persistent reservoirs and from human outbreaks of gastroenteritis (Danyluk et al., 2008; Greene et al., 2008; Uesugi et al., 2007). Potential routes by which *S. enterica* and enterovirulent *E. coli* colonize edible plant parts in the field and under greenhouse conditions have been investigated (Fletcher et al., 2013; Islam et al., 2004; Moyne et al., 2011; Park et al., 2012). Pre-harvest, microbiological quality of fresh produce is affected by the presence of native epiphytic microbiota (including phytopathogens), the types and levels of irrigation, and the use of soil amendments (Allard et al., 2014; Brandl, 2006, 2008; Brandl and Amundson, 2008; Franz and van Bruggen, 2008; Gu et al., 2013; Gutierrez-Rodriguez et al., 2012; Moyne et al., 2011; Park et al., 2012; Poza-Carrion et al., 2013). However, relatively little remains known about the impact of crop production practices on post-harvest susceptibility of raw fruits and vegetables to human pathogens. With this study we focused on the effects of N and K fertilization regimes on the "susceptibility" of tomato fruits to post-harvest proliferation of *S. enterica*.

The rationale for this study was two-fold. First, plant nitrogen status is well-known to be associated with the susceptibility of crops to phytopathogens (Snoeijers et al., 2000). Varying fertilization levels in the field or under controlled laboratory conditions

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http://dx.doi.org/10.1016/j.fm.2014.03.017
0740-0020/

affects physiological and biochemical properties of tomatoes rendering them more or less susceptible to phytopathogens, although fertilization-dependent defense responses appear pathosystem-specific (Hoffland et al., 2000; Snoeijers et al., 2000). Susceptibility of tomatoes to plant pathogenic bacteria (*Pseudomonas syringae* and *Xanthomonas* spp.) and to an obligately biotrophic ascomycete *Oidium lycopersicum* increased proportionately with the increase in nitrogen fertilization, while susceptibility to the vascular pathogen *Fusarium oxysporum* f.sp. *lycopersici* or a grey mold pathogen *Botrytis cinerea* either was not affected or decreased in response to increased nitrogen fertilization (Hoffland et al., 2000 and references therein). Phenolics and enzymes with defense functions tended to accumulate in tissues of tomatoes that experience nitrogen limitation (Benard et al., 2009; Stout et al., 1998). Although *Salmonella* is not considered to be a plant pathogen, it is reasonable to consider a possibility that increases in plant defense compounds or enzymes in response to varying levels of N and/or K fertilization would restrict proliferation of this human pathogen inside tomato tissues. Additionally, *Salmonella* proliferation inside tomato fruits is almost certainly impacted by the abundance of nutrients, which depends on the nitrogen status of the plant. An inverse relationship between accumulation of organic acids (or sugar/acid ratios) and phenolics in tomato leaf tissues in response to availability of nitrogen has been reported (Benard et al., 2009; Le Bot et al., 2009), however it is not known whether the presence of these compounds in tomatoes affects proliferation of *S. enterica* within plant tissues. Levels of nitrogen fertilization affected relative abundance of sucrose (not consumed by *S. enterica*) and glucose in tomato tissues (Abro et al., 2013; Mittelstrass et al., 2006), which may increase the availability of nutrients and promote proliferation of *Salmonella* within harvested fruit.

2. Materials and methods

2.1. Field production conditions

Seeds of tomatoes (cultivars Solar Fire and Sebring) were purchased from Siegers Seed Co. (Holland, MI) and Harris Co. (Rochester, NY). Transplants were produced in an environmental chamber on the University of Florida campus, and then planted in the field. Experiments were conducted in the Spring and Fall production seasons (that are typical for the temperate and subtropical climates of North and Central Florida) over two years in two geographic locations: Live Oak (30°18′07.22″; 82°53′58.865″) and in Citra (29°24′37.84″N; 82°10′12.14″W). The studies were conducted at Live Oak in the spring of 2011 and spring of 2012, and in Citra in the fall of 2012. Soil at the Live Oak site was a Lakeland fine sand (thermic, coated Typic Quartzipsamments), and the soil at the Citra site was a Gainesville loamy sand (hyperthermic, coated Typic Quartzipsamments).

Generally recommended practices for Florida tomato production were used for this research, including polyethylene-mulched raised beds, soil fumigation, drip irrigation, and pest control (Olson et al., 2012). A cover crop (15 cm tall) of rye (*Secale cereale* L.) was rototilled in preparation for tomato production. Soil tests were conducted prior to each production season using the Mehlich-1 soil testing method (Mehlich, 1953; Savoy, 2009). In Citra and Live Oak, the soil tested low in potassium (K) and high (>60 mg/kg extractable P) in phosphorus (P). At this concentration of P, the soil can supply all the needed P for the crop, so no P fertilizer was applied in either season. The experiment consisted of a factorial arrangement of fertilizer nutrient (N or K), rate of nutrient, and tomato cultivar. The main-plot was rate of nitrogen fertilizer, the sub-plot was rate of K fertilizer, and the sub–sub plot was tomato cultivar. Treatments were replicated three times in a randomized, complete-block design. Three levels of N and K fertilizer rate were used (168, 224, and 280 kg/ha N, and 140, 210, and 280 kg/ha K) in all possible combinations with the two cultivars. Twenty percent of the N and K was applied as dry fertilizer (from ammonium nitrate and potassium-magnesium sulfate) to the soil in the bed area and incorporated by roto-tilling before making the bed and applying the drip tubing and mulch (described below). Remaining N and K (ammonium nitrate plus potassium chloride) were injected through the drip irrigation system in six bi-weekly amounts during the growing season.

The soil at each site was formed into raised beds with 1.5 m between the centers of adjacent beds and the soil was fumigated with a mixture of 50% methyl bromide: 50% chloropicrin to control soil-borne pests and weeds. Pre-emergence herbicides were applied carefully to the soil surface in the alleys between beds to control weeds. Drip irrigation tubing (with emitters spaced 0.2 m apart applying 0.15 L/min/m^2) was applied to the surface of the beds approximately 0.2 m to the side of the middle of the bed. Black polyethylene mulch was applied to the beds for the spring crops and silver-on-black (silver side up) for the fall. Three weeks after fumigation, tomato transplants were placed through holes in the mulch. Tomato plants were placed in single rows on the mulched bed with 0.4 m between plants in the row. Planting dates were 17 March 2011, 12 March 2012, and 9 August, 2012. Plots consisted of a single row of tomatoes 8.2 m in length. Drip irrigation was applied under the mulch to maintain volumetric water content (measured by time domain reflectometry) at 8–10% (Munoz-Carpena, 2012). Early in the season, one irrigation event of 30 min per day was satisfactory to maintain optimal soil moisture. Irrigation frequency was increased to two 30-min runs per day as the crop developed and then finally to three 30-min runs per day as the fruit matured. During the season, fungicides, bactericides, and insecticides were applied as recommended by field scouting and consistent with commercial tomato production practices.

On three dates in spring of 2011 at Live Oak, 8 whole most recently matured (petiole plus blades) leaves (6th leaf from the tip of a branch) were collected from plants in each plot. The sampling dates for the spring 2011 crop were 19 April (first flower), 10 May (early fruit set), and 24 May (just prior to first harvest). Leaves were sampled in the spring 2012 crop on 12 April (first flower), 30 April (early fruit set), and 24 May (just prior to first harvest). Leaves were sampled in the fall 2012 crop on 4 September (first flowers) and 24 September (just before first harvest). Whole leaves were dried in a forced-air oven at 60 °C for three days and ground in a Wiley mill. A 0.2 g sample of a ground tissue was treated with the Kjeldahl digestion mixture and sulfuric acid on a heating block. We used the semi-automated colorimetric analysis (EPA Method 351.2) to determine nitrogen in total Kjeldahl nitrogen digests on an auto-analyzer (O'Dell, 1993).

Fresh petiole sap was analyzed for nitrate-N concentration for each of the sampling times. Three most-recently matured leaves were collected and stripped of the leaf blades. The petioles were chopped and the sap expressed with a hydraulic press. The sap was analyzed for nitrate-N concentration with a hand-held ion-specific electrode (Spectrum Technologies, Plainfield, IL). Results of sap analyses were expressed as mg/L fresh sap.

2.2. Tomato infections post-harvest

Tomatoes were harvest at various maturity stages. For the purpose of this analysis, they were further grouped into "unripe", "partially ripe" and "ripe". "Unripe" tomatoes were mature green (stage 1 of the USDA Color Classification Requirements, http://ucanr.edu/repository/a/a=83755) at field harvest and *Salmonella* infection and were either breakers, turning, pink or light red

(stages 2, 3, 4 or 5) upon completion of the 1 week-long incubation. “Partially ripe” refers for tomatoes that were mature green, breakers, turning or pink (stages 1, 2, 3 or 4) at field harvest and infection with *Salmonella*, but turned light red or red (stages 5 or 6) upon completion of the incubation. “Ripe” refers to tomatoes that were light red or red (stages 5 or 6) at field harvest and turned or stayed red during the incubation under the laboratory conditions. Harvested tomatoes were brought into the lab and inoculated with *Salmonella* through shallow wounds, typically within 2–24 h of the harvest, as previously described (Marvasi et al., 2013). For the inocula, the type strain *S. enterica* sv Typhimurium ATCC14028 or strains of *S. enterica* (Javiana ATCC BAA-1593, *S.* Montevideo LJH519, *S.* Newport C6.3, *S.* Braenderup 04E01347, 04E00783, 04E01556) linked to the human outbreaks of salmonellosis were individually grown overnight at 37 °C in LB broth with shaking. They were then washed twice in phosphate-buffered saline (pH 7.0), and the strains from the outbreaks were combined into a six-strain “cocktail” as suggested by the Framework for Evaluation of Microbial Hazards (Harris et al., 2012; Harris et al., 2013). These inocula were further diluted in sterile water and 3 μl of the suspension (containing between 100 and 1000 CFU) were spotted onto three shallow (~1 mm) wounds in tomato epidermis. Infected tomatoes were incubated at room temperature for a week. Upon completion of the incubation, tomatoes were macerated in an equal volume of PBS using a stomacher (Sevard) (200 r.p.m for 1 min) and the suspensions were plated onto a Xylose Lysine Deoxylate (XLD) agar (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37 °C–42 °C overnight. Proliferation was calculated by dividing the total CFU recovered from each tomato by the total CFU inoculated into each fruit. This allows to account for differences in tomato sizes and for the fact that the colonization of a tomato fruit by *Salmonella* is not even. The ratios were further subjected to the $\log_{10}$ transformation. XLD plates on which there were no *Salmonella* colonies upon completion of the incubation were treated based on the rules of Most Probable Number (MPN) analysis, i.e. the most probable number, rather than a zero, was used for the calculations. This is a more conservative approach.

2.3. Data analyses

The experimental data were analyzed as a Split-split-split-split plot design, with the whole plot nitrogen treatments arranged in a randomized complete block design with four blocks. The whole plot factor was “nitrogen fertilization” (with three levels), the split plot factor was “potassium fertilization” (with three levels), the split-split-plot factor was “cultivar” (with two levels), and the split-split-split-plot factor was “*Salmonella* strain” (with two levels). The experiment was replicated at two locations, and conducted over three time periods. Maturity was a discrete covariate measured on the tomato fruits over the one-week period from harvest to *Salmonella* inoculation. Tomatoes of commercial size were harvested twice per season, and since the same split-split-split-plots were not randomized over the seasons, we used a split-split-split-split-plot statistical design with repeated measures over seasons to analyze the data. Ten tomatoes for each block by nitrogen fertilization level by potassium fertilization level by cultivar by *Salmonella* strain by sampling time by production site were collected, and thus, the numbers of tomatoes represent subsamples, and are considered as nested levels of an unbalanced pseudo-replication factor. Main effects, two-way, and three-way interaction effects were included in the model while higher order interactions were not considered in the model. The significance of the main effects, two-way, and three-way interaction effects were tested using the (partial) F-tests of fixed effects. Mean separation for significant fixed effects or for effects for each level of other fixed effects of significant interactions in the model was performed using Tukey’s multiple comparison testing procedure and the corresponding letter grouping display.

Data analysis was performed using SAS software. Specifically, we fitted the following linear mixed effects model for the split-split-split-split-plot statistical design with repeated measures over seasons and pseudo-replications:

$$\begin{aligned} Y_{ijklsutr} = {} & \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \eta_l + \theta_s + \psi_u + \delta_{ijklsu} + \varphi_t \\ & + (\alpha\gamma)_{ik} + (\alpha\eta)_{il} + (\alpha\theta)_{is} + (\alpha\psi)_{iu} + (\alpha\varphi)_{it} + (\gamma\eta)_{kl} \\ & + (\gamma\theta)_{ks} + (\gamma\psi)_{ku} + (\gamma\varphi)_{kt} + (\eta\theta)_{ls} + (\eta\psi)_{lu} + (\eta\varphi)_{lt} \\ & + (\theta\psi)_{su} + (\theta\varphi)_{st} + (\alpha\gamma\eta)_{ikl} + (\alpha\gamma\theta)_{iks} + (\alpha\gamma\psi)_{iku} \\ & + (\alpha\gamma\varphi)_{ikt} + (\alpha\eta\theta)_{ils} + (\alpha\eta\psi)_{ilu} + (\alpha\eta\varphi)_{ilt} + (\alpha\theta\psi)_{isu} \\ & + (\alpha\theta\varphi)_{ist} + (\gamma\eta\theta)_{kls} + (\gamma\eta\psi)_{klu} + (\gamma\eta\varphi)_{klt} + (\eta\theta\psi)_{lsu} \\ & + (\eta\theta\varphi)_{lst} + (\theta\psi\varphi)_{lst} + \varepsilon_{ijklsutr}, \end{aligned}$$

where μ is the overall mean, α_i, γ_k, η_l, θ_s, ψ_u, and φ_t are the main effects of nitrogen fertilization, potassium fertilization, cultivar,

Table 1
F tests for the main effects and the two-way and three-way interaction effects of the factors nitrogen, potassium, tomato cultivar, tomato maturity, and time of harvest, *Salmonella* strains on susceptibility of the crop to proliferation of *Salmonella*.

Effect	*F* value	Prob > *F*[a]
Nitrogen	0.03	0.9672
Potassium	0.61	0.5438
Nitrogen × Potassium	1.03	0.4028
Tomato cultivar	0.62	0.4321
Tomato cultivar × Nitrogen	0.57	0.5727
Tomato cultivar × Potassium	0.72	0.4956
Tomato cultivar × Nitrogen × Potassium	0.57	0.6849
Salmonella strain	0.60	0.4386
Nitrogen × *Salmonella* strain	0.41	0.6632
Potassium × *Salmonella* strain	0.01	0.9947
Nitrogen × Potassium × *Salmonella* strain	1.49	0.2013
Tomato cultivar × *Salmonella* strain	0.01	0.9040
Tomato cultivar × Nitrogen × *Salmonella* strain	1.29	0.2745
Tomato cultivar × Potassium × *Salmonella* strain	2.43	0.0885
Time of harvest	351.80	<0.0001*
Nitrogen × Time of harvest	0.37	0.8983
Potassium × Time of harvest	2.38	0.0268*
Nitrogen × Potassium × Time of harvest	1.84	0.0366*
Tomato cultivar × Time of harvest	0.40	0.7535
Tomato cultivar × Nitrogen × Time of harvest	2.50	0.0205*
Tomato cultivar × Potassium × Time of harvest	0.15	0.9883
Salmonella strain × Time of harvest	9.03	<0.0001*
Nitrogen × *Salmonella* strain × Time of harvest	1.32	0.2459
Potassium × *Salmonella* strain × Time of harvest	1.93	0.0718
Tomato cultivar × *Salmonella* strain × Time of harvest	1.36	0.2522
Tomato maturity	96.13	<0.0001*
Tomato maturity × Nitrogen	1.24	0.2905
Tomato maturity × Potassium	0.57	0.6872
Tomato maturity × Nitrogen × Potassium	1.12	0.3495
Tomato maturity × Tomato cultivar	0.26	0.7685
Tomato maturity × Tomato cultivar × Nitrogen	1.86	0.1139
Tomato maturity × Tomato cultivar × Potassium	1.36	0.2452
Tomato maturity × *Salmonella* strain	0.12	0.8859
Tomato maturity × Nitrogen × *Salmonella* strain	1.27	0.2785
Tomato maturity × Potassium × *Salmonella* strain	0.68	0.6043
Tomato maturity × Tomato cultivar × *Salmonella* strain	0.07	0.9335
Tomato maturity × Time of harvest	10.41	<0.0001*
Tomato maturity × Nitrogen × Time of harvest	2.10	0.0141*
Tomato maturity × Potassium × Time of harvest	0.68	0.7726
Tomato maturity × Tomato cultivar × Time of harvest	4.33	0.0002*
Tomato maturity × *Salmonella* strain × Time of harvest	1.13	0.3399

[a] *F* value represents the value of the *F* test and Prob > *F* is the *p*-value of the *F* test for the corresponding effect. Values of 0.05 or less are considered evidence that the corresponding effect is significant (i.e., at least one effect is significant) and asterisk (*) indicates statistically significant effects at 0.05 nominal level.

Salmonella strain, maturity, and sampling time, β_j, $(\alpha\beta)_{ij}$, δ_{ijklsu} are the random effects of block, whole plot error, and split plot error, $(\alpha\gamma)_{ik}$, $(\alpha\eta)_{il}$, $(\alpha\theta)_{is}$, $(\alpha\psi)_{iu}$, $(\alpha\varphi)_{it}$, $(\gamma\eta)_{kl}$, $(\gamma\theta)_{ks}$, $(\gamma\psi)_{ku}$, $(\gamma\varphi)_{kt}$, $(\eta\theta)_{ls}$, $(\eta\psi)_{lu}$, $(\eta\varphi)_{lt}$, $(\theta\psi)_{su}$, and $(\theta\varphi)_{st}$ are the two-way interaction effects, $(\alpha\gamma\eta)_{ikl}$, $(\alpha\gamma\theta)_{iks}$, $(\alpha\gamma\psi)_{iku}$, $(\alpha\gamma\varphi)_{ikt}$, $(\alpha\eta\theta)_{ils}$, $(\alpha\eta\psi)_{ilu}$, $(\alpha\eta\varphi)_{ilt}$, $(\alpha\theta\psi)_{isu}$, $(\alpha\theta\varphi)_{ist}$, $(\gamma\eta\theta)_{kls}$, $(\gamma\eta\psi)_{klu}$, $(\gamma\eta\varphi)_{klt}$, $(\eta\theta\psi)_{lsu}$, $(\eta\theta\varphi)_{lst}$, and $(\theta\psi\varphi)_{lst}$ are the three-way interaction effects, where $\beta_j \sim N(0, {\sigma_\beta}^2)$, $(\alpha\beta)_{ij} \sim N(0, {\sigma_{\alpha\beta}}^2)$, $\delta_{ijkls} \sim N(0, {\sigma_\delta}^2)$ are the independent random effects corresponding to the random blocks, whole plot units, the split-split-split-split units, and $\varepsilon_{ijklsutr} \sim N(0, {\sigma_\varepsilon}^2)$ are the independent random (pseudo-replication) errors.

The mixed effects linear model was fitted in SAS/GLIMMIX. We first identified the significant effects using the results of the partial *F* tests at a nominal type I error rate (i.e., probability to wrongly reject the null hypothesis) of 0.05. Then, Tukey's multiple comparison procedure (including the letter grouping from the lines display) was performed to separate the model-predicted balanced means for the significant effects in the model. This approach to post-hoc analysis enabled us not only to identify significant differences between various treatment means, but also to assess whether the treatment mean differences were practically significant. Goodness-of-fit tests for the fitted model were conducted in SAS/UNIVARIATE by testing the normality assumption of the standardized residuals using the Kolmogorov–Smirnov and Cramer–von Mises tests of normality. Since there was not a very strong evidence against the normality assumption of the standardized residuals, the statistical conclusions reported here have a high degree of accuracy and precision.

2.4. Growth in the presence of tomato phenolics

To determine if tomato flavonoids affect *S. enterica* sv Typhimurium directly, its growth in the presence of quercetin, rutin, and kaempferol (all purchased from Sigma–Aldrich, St Luois, MO) was tested. Stock solutions of flavonoids were prepared in DMSO. Flavonoids were further diluted in separate Erlenmeyer flasks, each with 20 ml of LB broth to result in 350 mg L^{-1}, 80 mg L^{-1}, 220.2 g L^{-1} of rutin, quercetin and kaempferol (respectively), which corresponds to 10 times the concentration of these flavonoids in tomato fruits at different maturity stages (Dumas et al., 2003). These media were then inoculated with a diluted overnight culture of *Salmonella* Typhimurium, and incubated shaken at 250 r.p.m. at 37 °C. Growth was monitored by dilution plating on LB agar at time 0, 4, 6, 24 and 72 h.

3. Results and discussion

3.1. Overall trends: effects of field crop production variables on susceptibility of tomatoes to post-harvest infections with S. enterica

Even though some studies demonstrated that *Salmonella* involves its virulence genes in the interactions with plants, and that under some conditions it can elicit disease-like signs in plants (Schikora et al., 2011), it is not commonly considered a phytopathogen (Barak and Schroeder, 2012). Therefore, for the purpose of this article, we broadly define "susceptibility" of tomatoes as being conducive to proliferation of *Salmonella* following an infection into a shallow wound in the fruit epidermis. Colonization of fruits through natural or artificial openings in fruits is considered by the US FDA to be a major route of tomato infection (Anonymous, 2013).

Based on the *F*-test for the main effects for all fixed factors, varying levels of nitrogen fertilization ($F = 0.03$, $P = 0.967$) or potassium fertilization ($F = 0.61$, $P = 0.544$) alone did not affect overall susceptibility of tomatoes to infections with *S. enterica* (Table 1, Fig. 1, Supplemental Table 1). The main effects of the tomato cultivar ($F = 0.62$, $P = 0.432$) and *Salmonella* strain ($F = 0.60$, $P = 0.4386$) were not significant, while the main effects of the harvest time ($F = 351.80$, $P < 0.0001$) and tomato maturity ($F = 96.13$, $P < 0.0001$) were highly significant. The effects of tomato maturity on *Salmonella* growth inside tomatoes are consistent with the reports that red tomatoes were significantly more conducive to proliferation of *Salmonella* than green tomatoes (Shi et al., 2007; Marvasi et al., 2013) and that the gene expression in *Salmonella* changes in response to specific metabolites present within immature tomato fruit (Marvasi et al., 2013; Noel et al., 2010).

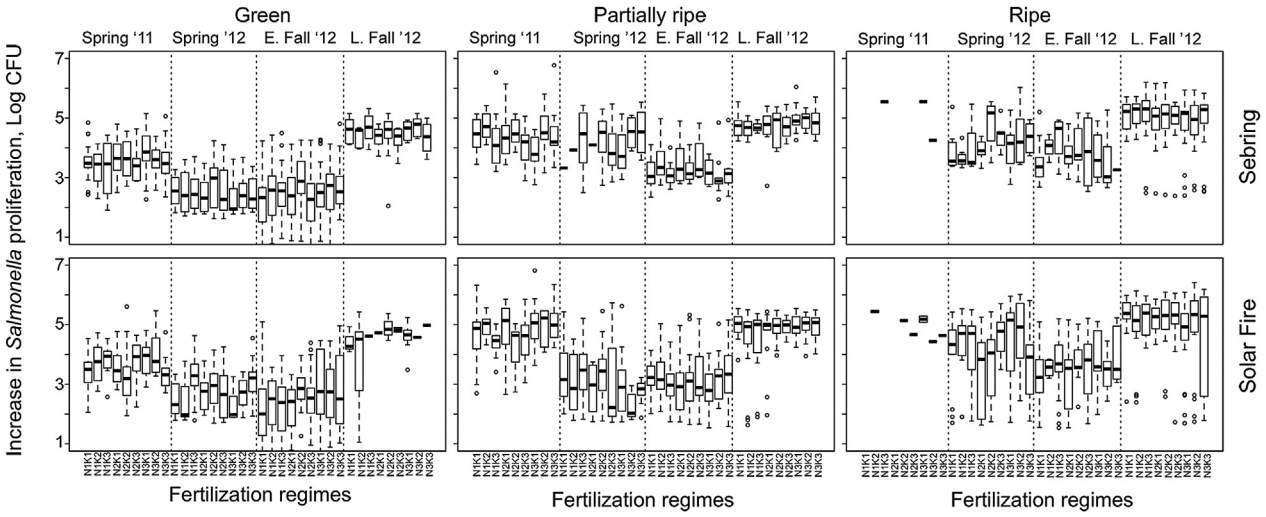

Fig. 1. Post-harvest proliferation of *Salmonella* in ripe, partially ripe and un-ripe tomatoes grown under different fertilization regimes. Tomatoes (cultivars Sebring and Solar Fire, indicated on the right *y*-axis) were grown under differential fertilization regimes: N1 = 168, N2 = 224 (recommended), N3 = 280 kg/ha; K1 = 168, K2 = 252 (recommended), K3 = 336 kg/ha. Four independent samplings (top *x*-axis) were conducted: once in Spring 2011 and Spring 2012, and twice in Fall 2012. At each sampling, at least 55 tomatoes from each treatment were harvested and infected with $\sim 10^2$ CFU of *S.* Typhimurium 14028 or the cocktail of the six strains of *S. enterica*. Upon completion of a 1-week incubation, *Salmonella* cells were recovered and an increase in proliferation was calculated as Log (CFU per $fruit_{HARVEST}$/CFU per $fruit_{INOCULUM}$) and plotted on the *y*-axis for each tomato cultivar. Data for infections with both types of inocula are shown. In box plots, boundaries of boxes include the lower and upper quartiles, thick lines within the box are the medians and whiskers indicate the degree of dispersion of the data. Outlier data are shown as dots.

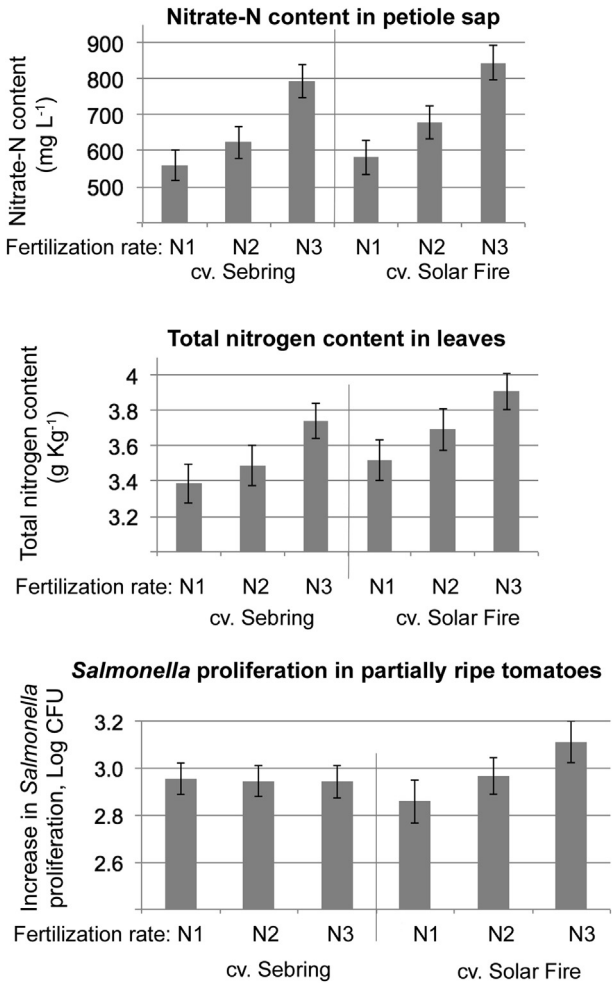

Fig. 2. Nitrogen tissue-level differences in tomatoes and proliferation of *Salmonella*. Petiole sap and leaves were collected from 8 plants in Spring 2011 and Spring and Fall 2012 and analyzed as described in Materials and Methods. Levels of nitrogen fertilization are shown on the *x*-axis (N1 = 168, N2 = 224 (recommended), N3 = 280 kg/ha), all shown samples were collected from plants that were grown under the recommended potassium fertilization (210 kg/ha K).

Statistically significant two-way and three-way interactions were as follows: potassium fertilization levels × harvest time ($F = 2.38$, $P = 0.027$), *Salmonella* strain × harvest time ($F = 9.03$, $P < 0.0001$), tomato maturity × harvest time ($F = 10.41$, $P < 0.0001$), nitrogen fertilization levels × potassium fertilization levels × harvest time ($F = 1.84$, $P = 0.037$), tomato cultivar × nitrogen fertilization level × harvest time ($F = 2.50$, $P = 0.021$), tomato maturity × nitrogen fertilization levels × harvest time ($F = 2.10$, $P = 0.014$), and tomato maturity × tomato cultivar × harvest time ($F = 4.33$, $P = 0.0002$), respectively (for the results of all *F*-tests, see Table 1). Some of these results will be discussed below in detail.

The effects of potassium were not significant for seasons A ($F = 0.69$, $P = 0.503$) and D ($F = 0.04$, $P = 0.965$), but were significant for seasons B ($F = 8.13$, $P = 0.0004$) and C ($F = 3.20$, $P = 0.04$), with an increase in potassium level associated with an increase in the proliferation of *S. enterica* (based on Tukey mean separation of the effects of potassium for each season). This suggests that when *Salmonella* proliferation is low (e.g., seasons B and C), lower levels of potassium are associated with lower *Salmonella* proliferation levels. While the *F*-tests for simple effects of two-way interaction of nitrogen × potassium for each season show that the interaction effects were not significant for all seasons (A: $F = 0.53$, $P = 0.83$; B: $F = 2.34$, $P = 0.20$; C: $F = 1.10$, $P = 0.478$; D: $F = 0.89$, $P = 0.56$), Tukey mean separation identifies a nitrogen × potassium treatment that has a significantly lower proliferation than the largest mean for season B ($N = 2$ and $K = 1$). The effects of the *Salmonella* strain were not significant for seasons A, B, and C (A: $F = 0.05$, $P = 0.83$; B: $F = 0.41$, $P = 0.52$; C: $F = 3.29$, $P = 0.07$), while the strain effects were significant for season D ($F = 26.73$, $P < 0.0001$), with an average decrease of 0.42 in proliferation for the cocktail of the outbreak strain vs the type strain of *S.* Typhimurium 14028 ($t = -5.17$, $P < 0.0001$).

3.2. Effects of differences in tissue nitrogen levels on susceptibility of tomatoes to infections with S. enterica

Differences in the nitrogen and potassium levels in petiole sap and leaves were determined to establish whether the imposition of different fertilization regimes led to the differences in the tissue levels of these plant nutrients. We then tested whether tissue-level differences in N or K correlated with the susceptibility to *Salmonella*. Even though levels of K in petioles and leaves generally correlated with the treatments, we did not observe a relationship with the susceptibility to *Salmonella* (data not shown). A correlation between tissue levels of total nitrogen, Nitrate-N in petiole sap and susceptibility to *Salmonella* were observed for partially ripened tomatoes of cv. Solar Fire, but not Sebring, even though both varieties accumulated more nitrogen in their vegetative tissues in response to the increase in N supplied with the fertilizer treatment (Fig. 2). The mechanism behind this observation is not yet known, however, it has been reported that tomato varieties responded to varying levels of nitrogen nutrition by producing different levels of flavonoids and related phenolics (Larbat et al., 2012).

To test the hypothesis that tomato flavonoids were responsible for these observed differences in the proliferation of *Salmonella*, the ability of this pathogen to grow *in vitro* in the presence of tomato

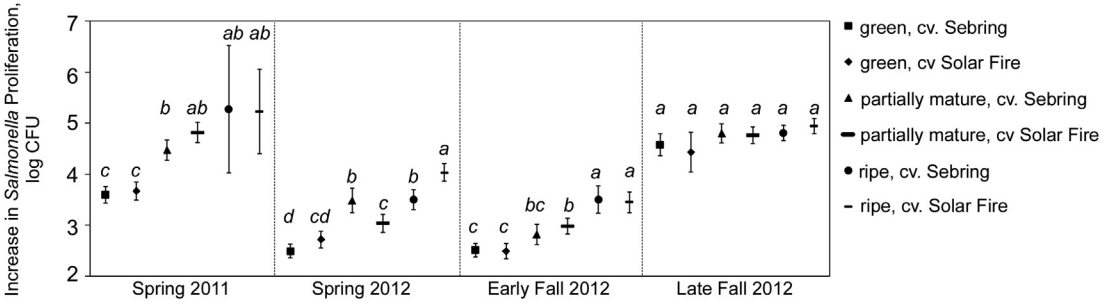

Fig. 3. Effect of tomato maturity, genotype and time of harvest on susceptibility to *Salmonella*. 95% Confidence intervals for the predicted means of *Salmonella* proliferation in tomatoes of two varieties, sampled at different maturity stages during the four samplings. Letters indicate statistical groupings within each sampling.

phenolics was tested. When incubated in LB broth with rutin, quercetin and kaempferol, *Salmonella* reached the same final population densities as when grown in LB broth with the carrier solvent (DMSO) only, reaching 7.54 ± 0.06, 7.84 ± 0.15, 7.87 ± 0.16 $\log_{10}$ cfu/mL (respectively, for rutin, quercetin and kaempferol) compared to 7.53 ± 0.03 $\log_{10}$ cfu/mL for a control. Growth rates in the cultures treated with the phenolics were indistinguishable from the control exposed to DMSO only (data not shown). Therefore, even if the accumulation of phenolics in tomato fruits of the cv. Solar Fire was dependent on the levels of nitrogen fertilization, phenolics were not likely responsible for the observed differences in *Salmonella* proliferation.

Interestingly, a trend of increased proliferation within tomato leaves in response to N tissue-level differences was observed for *P. syringae* (Hoffland et al., 2000). Whether or not *P. syringae* and *Salmonella* rely on similar strategies for colonization of plants will be important to determine, and this comparison with the *P. syringae* plant-specific gene expression (Boch et al., 2002) will be possible as soon as *Salmonella* gene expression within plant tissues is similarly documented.

3.3. Seasonal variability and susceptibility of tomatoes to infections with S. enterica

Season-to-season variability in the susceptibility of crops to colonization by *Salmonella* and enterohemorrhagic *E. coli* pre- and post-harvest have been previously reported (Gutierrez-Rodriguez et al., 2012; Marvasi et al., 2013). *Salmonella* proliferation was the highest in fruits harvested in Spring 2011 and Fall 2012 (harvests A and D, Fig. 3), with individual 95% confidence intervals for the average proliferation of (4.22,4.88) and (4.49,5.03), respectively, and with the lowest average proliferation levels during the seasons B and C, with 95% confidence intervals for average proliferation of (2.93,3.57) and (2.69,3.31), respectively (Fig. 3).

Weather conditions within a month prior to harvests were different in each of the experimental seasons (Fig. S1) and weather parameters suggested as consequential to the proliferation of human pathogens in the field (Harris et al., 2012) are discussed below. Average daily temperatures one month before the harvest in Spring 2011, Spring 2012 and Fall 2012 were 25.0 °C, 24.1 °C, and 21.3 °C, respectively (Fig. S1). It is of note, however, that the last harvest in Fall 2012 was preceded by a drop in temperature to 1.6 °C. During these production seasons, average relative humidity was 70.1, 75.4, and 82.1%; and total precipitation was 4.1, 41.3, and 14.4 (cm m^{-2}). Average total radiant flux was 21.9, 18.9, and 13.9 (MJ m^{-2}) in these production seasons. Therefore, the harvests in which the tomato crops were the most susceptible to proliferation of *Salmonella* were those with low rainfall. Our parallel study testing the effects of varying levels of irrigation on the susceptibility of tomatoes and peppers to infections with *S. enterica* revealed that even though water congestion of green (and not red) fruit led to an increased susceptibility of tomatoes to infections with *S. enterica* these seasonal differences are not due to the amount of the rainfall per se (Marvasi et al., 2013).

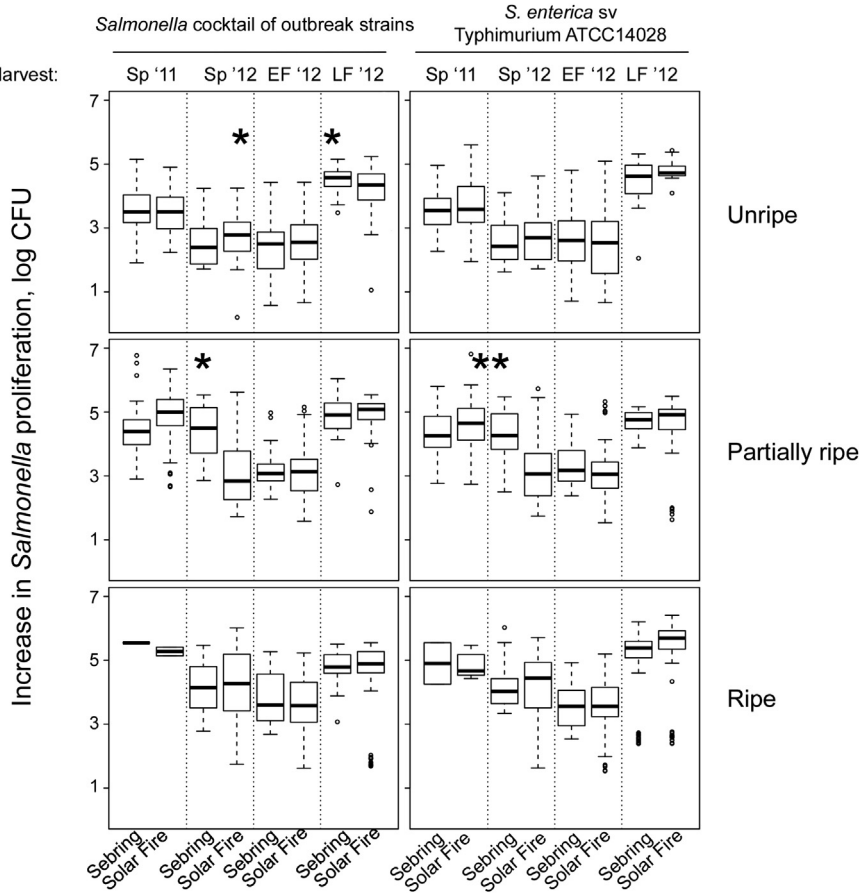

Fig. 4. Post-harvest proliferation of *Salmonella* in tomatoes of different varieties. The effect of tomato genotype on post-harvest proliferation of *Salmonella* was assessed in three seasons and presented as a cumulative data in each season. Within each sampling, statistical significance was assessed using pair-wise comparisons. Asterisks indicate significant differences among the samples within the same panel.

3.4. The role of plant genotype in susceptibility of the field crop

Under the laboratory conditions, endo- and epiphytic populations of human pathogens reached different levels in different crops and different varieties of the same crop, however, results of the field studies were more nuanced (Barak et al., 2011; Gutierrez-Rodriguez et al., 2012; Klerks et al., 2007; Shi et al., 2007; Marvasi et al., 2013). Only two tomato varieties were included in this study, as it was not designed as a comprehensive screen of the susceptibility of all tomato genotypes to *Salmonella* proliferation. The main and interaction effects of cultivar and season were not significant; the main effects of tomato maturity were significant, with 95% confidence for the average proliferation for immature, partially mature, and mature tomatoes of (3.03,3.67), (3.60,4.27), and (4.10,4.66), respectively. When *Salmonella* genotype was also considered, significant interactions between the tomato cultivar and the fruit maturity were observed for all harvests (A: $F = 29.61$, $P < 0.0001$; B: $F = 52.25$, $P < 0.0001$; C: $F = 23.09$, $P < 0.0001$; and D: $F = 2.38$, $P = 0.037$ (for more details, see Figs. 3 and 4)). Even though only two tomato cultivars were used in this study, further screens of different tomato varieties could reveal those that are consistently more resistant to at least some genotypes of *Salmonella*. The results of this field study are also informative in that even though strong statistically significant differences could be observed in one season, they may not be reproducible under a different set of environmental conditions, even in the same geographic location.

Acknowledgments

This research was supported by Florida Department of Agriculture/Specialty Block Grant contract # 16858 and by USDA NIFA-AFRI grant # 2011-67017-30127. ASG was supported by the McKnight Fellowship. We thank C. Chen, M. Carrazana, M. Farias, A. Aruca, M.H. Moraes, K. Jenkins, and D. Lucas for their help with tomato processing during harvest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.03.017.

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